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Expression of Immune Regulatory Molecules in Epstein-Barr Virus-Associated Nasopharyngeal Carcinomas with Prominent Lymphoid Stroma

Evidence for a Functional Interaction between Epithelial Tumor Cells and Infiltrating Lymphoid Cells

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Undifferentiated nasopharyngeal carcinomas (UNPC) are characterized by an association with Epstein-Barr virus and an abundant lymphoid stroma. The role of this lymphoid stroma is uncertain but is mostly thought to represent an immune response against viral or tumor antigens. We have analyzed the expression of immune regulatory receptor/ligand pairs in snap-frozen biopsies of 20 UNPCs. All cases were Epstein-Barr virus positive and the virus-encoded latent membrane protein, LMP1, was expressed in 6 cases. By immunohistochemistry, we have demonstrated the expression of CD70 and CD40 in the tumor cells of 16 and 18 cases, respectively. Infiltrating lymphoid cells expressing CD27, the CD70 receptor, and the CD40 ligand were present in all cases. The Bcl-2 protein was detected in 17 cases. Unexpectedly, tumor cells of 5 cases expressed at least one member of the B7 family (CD80, CD86, and B7-3) and many lymphoid cells expressing the corresponding counter-receptor, CD28, were detected in all cases. Interestingly, 5 of 6 LMP1-positive cases also expressed B7, whereas all 14 LMP1-negative cases were also B7 negative. Our results indicate that T cells and carcinoma cells communicate in the microenvironment of UNPCs and suggest that the presence of a lymphoid stroma may be a requirement for UNPC growth at least in certain stages of tumor development. (Am J Pathol 1995, 147:1152–1160)

The Epstein-Barr virus (EBV) is associated with several lymphoid malignancies, eg, Burkitt's lymphoma, and Hodgkin's disease.[1–3] However, the tumor showing the most consistent association with the virus is undifferentiated nasopharyngeal carcinoma (UNPC).[1,4] UNPCs harbor monoclonal viral genomes in virtually all tumor cells, indicating that EBV infection has taken place before expansion of the malignant cell clone.[4–7] Furthermore, EBV is detectable in virtually all UNPCs, suggesting that EBV infection is a rate-limiting step in the pathogenesis of UNPC.[4–7] A potential etiological role for EBV in the development of UNPC is further underlined by the detection of some viral gene products in the tumor cells. The EBV-encoded small nuclear RNAs (EBERs) and the virus-encoded nuclear antigen (EBNA-1) are expressed in all cases[6–10] and the latent membrane protein (LMP)-1 is detectable in a proportion of cases.[6,8–10] The latter observation is important because LMP-1 is the only viral gene product with transforming ability *in vitro*.[11] Also, LMP-1 can induce or increase the expression of lymphocyte activation molecules (CD23, CD30, CD70, and CD71) and adhesion molecules (CD54 and CD58) in certain B cell lines.[12–14] In epithelial cells, LMP-1 has been

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shown to inhibit differentiation and to increase the expression of the CD40, CD54, and CD70 antigens.[15–17] The latter observation is particularly intriguing because we have previously demonstrated that CD70 is expressed in most UNPC cases.[6,17]

A characteristic morphological feature of UNPCs is the presence of an intense lymphoid stroma.[18,19] This consists of many lymphoid cells, but eosinophils may be prominent in some cases. The lymphocytes are mainly T cells with CD4 positive cells and CD8 positive cells present in varying proportions.[20–22] Although some of these T cells display an activated phenotype, impaired T cell receptor- and CD2-mediated activation pathways have been observed.[20,21,23] The significance of the lymphoid stroma in UNPCs is uncertain, but most previous studies have suggested that it may represent an immune response to virus or tumor antigens expressed in the tumor cells.[20–23] Expression of major histocompatibility complex class II in UNPC biopsies as well as expression of interleukin-1 and other inflammatory cytokines in some UNPC cell lines has been demonstrated, suggesting that the tumor cells may contribute to the presence of the T cell infiltrate.[22,24,25] Indeed, it has been speculated that UNPC growth may require the presence of T cells at least at some stages during tumor development.[24]

To investigate further the significance of the lymphoid stroma in UNPCs, we have examined the expression of certain well defined immunoregulatory receptor/ligand pairs in UNPCs.

Materials and Methods

Tissues

Snap-frozen biopsies from 20 UNPCs from Beijing and Hong Kong were included in this study. The diagnosis of UNPC was confirmed in all cases by examination of hematoxylin and eosin (H&E)-stained sections and by immunohistochemistry, which revealed the expression of cytokeratins by the tumor cells in all cases.

In Situ *Hybridization*

In situ hybridization with probes specific for the EBV-encoded small nuclear RNAs, EBER-1 and EBER-2, was employed to assess the EBV status of the tumor cells. ^{35}S-Labeled RNA probes were generated and *in situ* hybridization was carried out as described previously.[6] Bound probes were detected by autoradiography as described.[6]

Table 1. *Monoclonal Antibodies*

Clone	Specificity	Source
BB-1	CD80/ B7–3	Becton Dickinson, Oxford, UK
BU63	CD86	Dr. D. Hardie, Birmingham, UK
124	Bcl-2	Dako, High Wycombe, UK
MT910	CD2	Dako
MT310	CD4	Dako
DK25	CD8	Dako
HD37	CD19	Dako
YTH913.12	CD28	Serotec, Oxford, UK
CD27/2	CD27	Bradsure Biologicals, Loughborough, UK
G28.5	CD40	Accession No. HB-9110, ATCC, Rockville, MD
M90	CD40L	Dr. R. Armitage, Immunex, Seattle, WA
Ki-24	CD70	Prof. H. Stein, Berlin, Germany
CS1–4	EBV LMP1	Dako

ATCC, American Type Culture Collection.

Immunohistology

Monoclonal antibodies (MAbs) used in this study are summarized in Table 1. Cryostat sections (6-μm) were put onto aminopropyltriethoxy silane (APES) coated slides and air dried overnight. Tissue sections were fixed in acetone for 10 minutes at room temperature (RT) before application of immunohistochemistry. Sections were incubated with appropriately diluted MAbs for 30 minutes at RT. Bound primary antibodies were then detected by a standard alkaline phosphatase-anti-alkaline phosphatase (APAAP) method.[26] Sections were incubated with rabbit anti-mouse immunoglobulins (Dako, High Wycombe, UK) for 30 minutes at RT followed by incubation with APAAP complex (Dako) for 30 minutes at RT. Incubations with rabbit anti-mouse immunoglobulin and with APAAP complex were then repeated twice for 10 minutes each to increase sensitivity of the staining. Immobilized alkaline phosphatase was visualized with naphthol AS MX phosphate and Fast Red TR salt followed by hematoxylin counterstaining.

Results

Phenotype of UNPC Tumor Cells

All 20 UNPC cases included in this study were EBV positive as assessed by *in situ* hybridization with EBER-specific RNA probes (Table 2). Expression of the LMP-1 gene product in UNPC tumor cells was demonstrated in 6 cases (30%; Table 2), in agreement with our previous results.[6]

Table 2. *Phenotype of Undifferentiated Nasopharyngeal Carcinomas*

Case	EBERs	LMP-1	Bcl-2	CD 40	CD 70	CD80/ B7–3	CD 86
1	+	–	+	+	+	–	–
2	+	+	+	+	+	–	–
3	+	+	+	+	–	+	+
4	+	+	+	+	+	+	–
5	+	–	+	+	–	–	–
6	+	+	+	+	+	+	+
7	+	–	–	–	–	–	–
8	+	–	+	+	+	–	ND
9	+	–	+	+	+	–	–
10	+	–	+	–	+	–	–
11	+	+	+	+	+	–	+
12	+	+	–	+	+	+	+
13	+	–	+	+	+	–	–
14	+	–	+	+	+	–	–
15	+	–	+	+	+	–	ND
16	+	–	+	+	+	–	–
17	+	–	+	+	+	–	–
18	+	–	+	+	+	–	–
19	+	–	+	+	+	–	–
20	+	–	–	+	–	–	–

ND, not done.

Expression of the CD70 antigen was noticed in 16 of 20 UNPC cases (80%; Table 2), consistent with previously published results.[6,17] In CD70 positive cases, strong expression of this antigen in virtually all tumor cells was observed (Figure 1). This was confirmed by comparison with serial sections stained with an anti-cytokeratin antibody.

The CD40 antigen was expressed by virtually all tumor cells in 18 of 20 cases (90%; Table 2 and Figure 2A). Expression of this molecule was also seen in basal epithelial cells of non-neoplastic nasopharyngeal mucosa present in some of the UNPC biopsies. Bcl-2 expression in the UNPC tumor cells was observed in 17 of 20 cases (85%; Figure 2B). Expression of this protein was also seen in normal basal epithelial cells. Although in most cases the tumor cells expressed both CD40 and Bcl-2, 1 case was negative for both antigens (case 7, Table 2), 1 case was Bcl-2 positive but CD40 negative (case 10, Table 2), and 2 cases were Bcl-2 negative and CD40 positive (cases 12 and 20, Table 2).

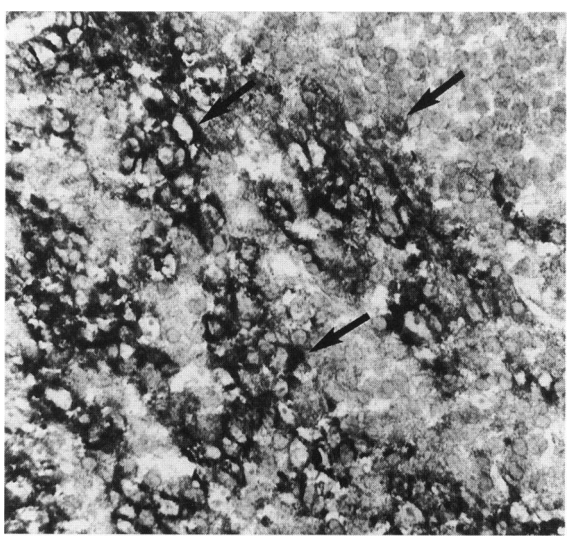

Figure 1. *Expression of the CD70 antigen is seen in an undifferentiated NPC (case 11). Note the dark staining of tumor cells* (arrows), *whereas admixed reactive cells are negative. An identical staining pattern was observed with cytokeratin-specific reagents on serial sections of the same case. APAAP method; hematoxylin counterstaining; magnification, × 350.*

Staining of epithelial tumor cells with the BB-1 MAb was observed in 4 of the 20 cases (20%; Table 2 and Figure 3, A and B). The staining pattern observed was patchy and often appeared to be stronger at the margins of tumor cell clusters. With a B7–2 (CD86)-specific MAb, staining of tumor cells was seen in 4 of 18 cases (22%) available for analysis (Table 2 and Figure 3, C and D), and 3 cases showed staining with both antibodies; thus, a total of 5 cases (25%) expressed at least one member of the B7 family. Interestingly, expression of B7 appeared to correlate with LMP-1 status. All 4 cases stained with the BB-1 MAb were LMP-1 positive, and 1 of the 2 LMP-1-positive/BB-1-negative cases expressed the CD86 antigen. Thus, 5 of 6 LMP-1 positive cases expressed one or more members of the B7 family, whereas none of the 14 LMP-1 negative cases showed B7 expression (Table 2). B7 expression was not noticed in normal nasopharyngeal mucosa present in some of the biopsies.

CD27, CD28, and CD40 ligand (CD40-L) were not expressed by the epithelial tumor cells.

Phenotype of Tumor-Infiltrating Cells

A prominent lymphoid stroma was noticed in all biopsies except one (case 9). This case did not show any striking phenotypic differences from the other cases (Table 2). Immunohistology with a CD2 MAb revealed a predominant T cell infiltrate, but variable numbers of B cells as identified with a CD19 MAb were also noticed. A more detailed analysis showed that CD8 positive and CD4 positive T cells were present in varying proportions. In contrast to one previous study, we did not find a significant predominance of CD4-positive cells over CD8-expressing cells (Figure 4, A and B). However, exact quantitative assessment is difficult by immunohistochemistry and we cannot exclude that subtle differences do exist.

In all 20 cases, many CD27 positive reactive cells were noticed. These were located mainly around the

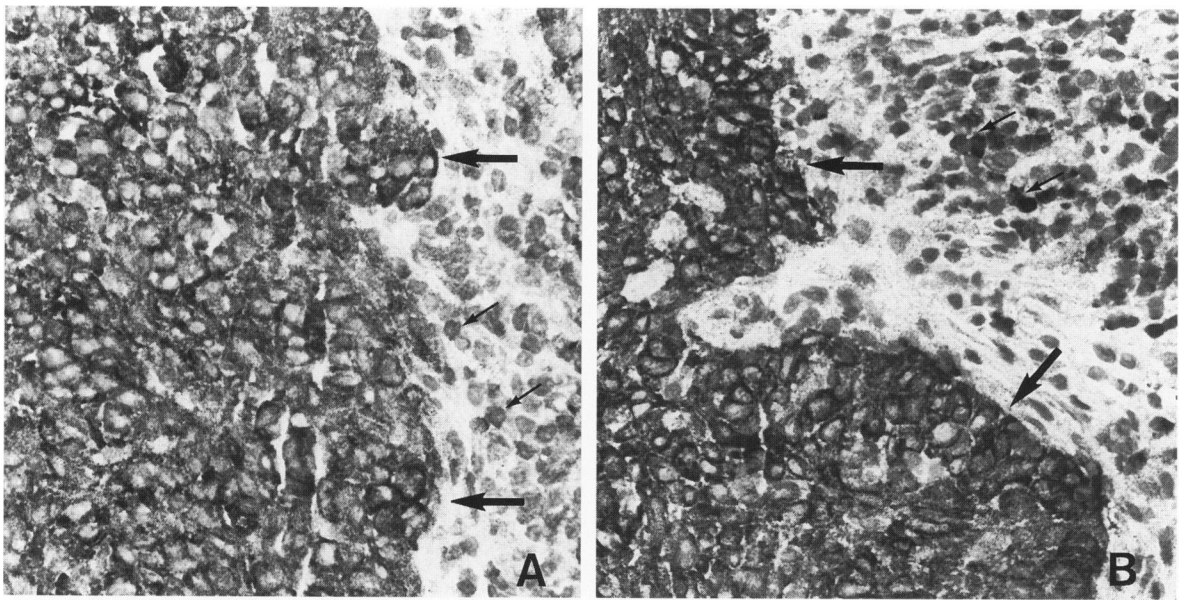

Figure 2. *Staining of virtually all tumor cells is seen in an undifferentiated NPC (case 1) with a CD40 MAb* (**A**). *Labeling of some lymphoid cells is also seen in the* right part *of this figure. The same case also shows strong expression of the Bcl-2 protein in the tumor cells* (**B**). *Some lymphoid cells in the* upper right corner *of this figure also express Bcl-2. In both figures,* long arrows *delineate the margin of the tumor cell clusters in the* left part *of the figures and* short arrows *indicate labeled lymphoid cells. APAAP method; hematoxylin counterstaining; magnification, × 350.*

tumor cell clusters but were also seen admixed with the tumor cells (Figure 5A).

Staining of variable numbers of lymphoid cells and dendritic cells was noticed in all cases with the BB-1 and BU63 MAbs. Moderately strong staining of scattered lymphoid cells was observed with the CD28-specific MAb (Fig. 5B).

Expression of CD40-L was seen in variable numbers of scattered lymphoid cells in all cases (Figure 5, C and D). CD40 and the Bcl-2 protein were expressed in many lymphoid cells in all biopsies (Figure 2, A and B).

Discussion

UNPCs are characterized by a consistent association with EBV and by the presence of an intense lymphoid stroma in most cases. The significance of this lymphoid stroma is uncertain but could be explained as follows. (1) It may represent pre-existent lymphoid tissue that is abundant in the normal nasopharynx.[18] (2) It may represent an immune response directed against tumor or EBV-encoded antigens expressed by the carcinoma cells.[20–23] (3) The lymphoid cells may contribute to optimal tumor cell growth, eg, through the elaboration of cytokines and other growth factors.[24]

A lymphoid stroma may be absent from metastatic as opposed to primary UNPCs, supporting the first hypothesis.[18] However, a lymphoid stroma is also usually absent from primary squamous cell NPCs.[19] Furthermore, undifferentiated carcinomas with a lymphoid stroma may arise at sites that do not normally contain lymphoid tissue, eg, stomach, skin, and uterine cervix.[27–29] Thus, although the lymphoid cell infiltrate found in UNPC biopsies may in parts represent pre-existent lymphoid tissue, it seems unlikely that this accounts for the close admixture of tumor cells with infiltrating activated T cells. Indeed, most authors appear to favor an immune function aimed at control of tumor cell growth.[20–23]

It has been suggested that the expression of interleukin-1 and other cytokines by UNPC cells could induce recruitment of T cells into the tumor where they might contribute to the tumor cell growth.[24,25] To address this problem, we have studied the expression of three receptor/ligand pairs in UNPC tumor cells and in infiltrating lymphoid cells.

We have confirmed our previous observation that CD70 is expressed in the tumor cells of most UNPCs.[6,17] CD70 is a member of a cytokine family with homologies to tumor necrosis factor.[30,31] CD70 expression in peripheral blood lymphocytes can be induced to high levels by activation or infection with viruses such as EBV and HTLV-1.[14] CD70 is also expressed in activated lymphoid cells in certain immunological reactions and in some lymphomas, most notably Hodgkin's disease.[14] CD70 is not detectable in normal epithelial cells, but it is inducible by LMP-1 in the immortalized keratinocyte cell line

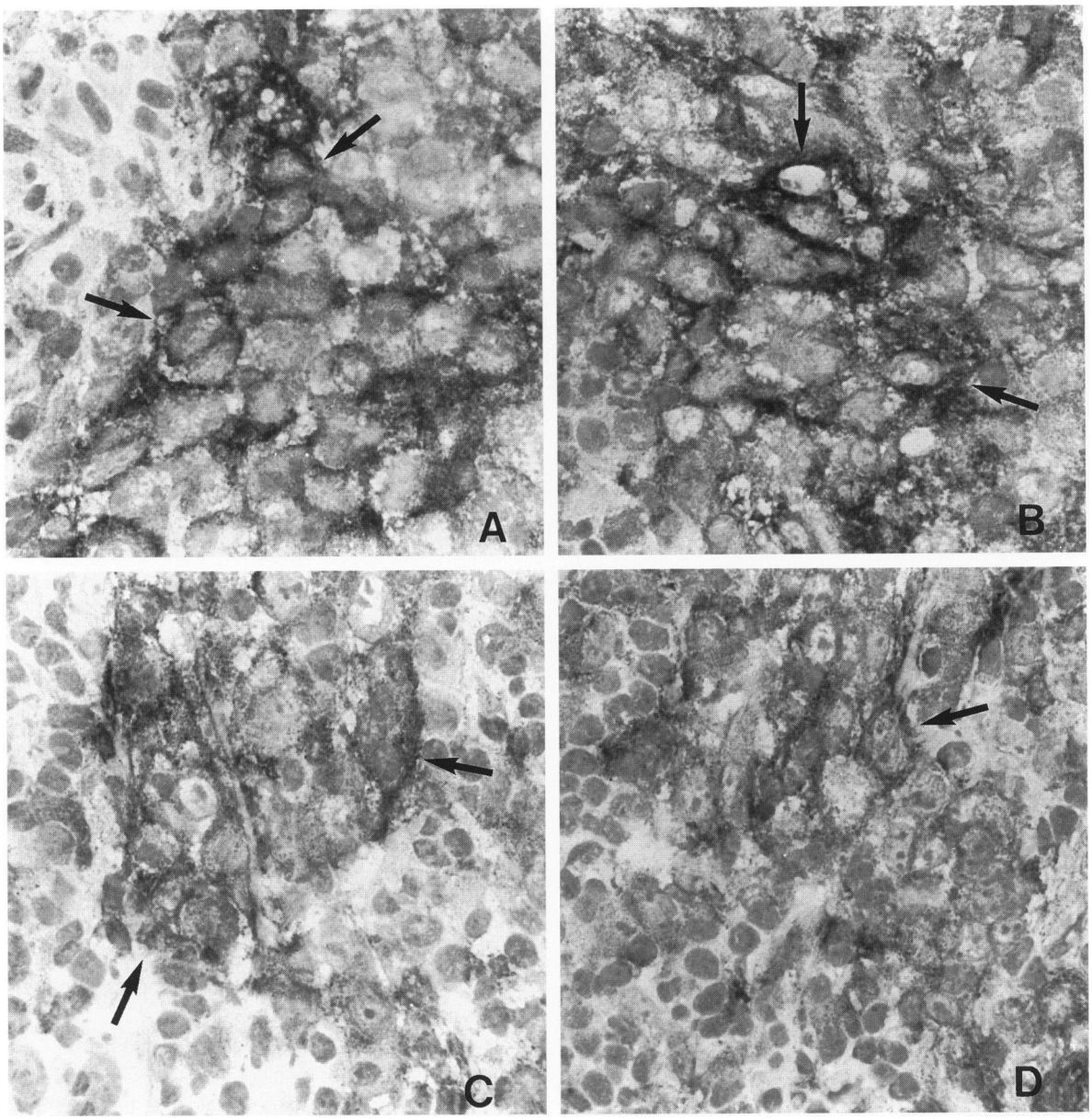

Figure 3. *B7 expression in undifferentiated NPCs. Patchy staining of epithelial tumor cells is seen in cases 11* (**A** *and* **B**) *and 12* (**C** *and* **D**) *with MAb BB-1 directed against B7–1* (*CD80*) *and B7–3* (**A** *and* **B**) *and with MAb BU63 directed against the B7–2* (*CD86*) *antigen* (**C** *and* **D**). **Arrows** *indicate labeled tumor cells. APAAP method; hematoxylin counterstaining; magnification,* × *700.*

RHEK-1.[17] The frequent expression of CD70 in UNPCs is thus unusual and may relate to the presence of EBV. CD70 functions as the ligand for CD27, a member of the tumor necrosis factor receptor family.[30,31] CD27 is expressed in medullary thymocytes, peripheral blood T cells, and mature B cells, including germinal center cells.[32] Ligation of CD27 may provide a co-stimulatory signal that is required for optimal proliferation of activated T cells.[32] Thus, the expression of CD70 in most UNPCs, together with the presence of many CD27 positive reactive cells in the lymphoid stroma, suggests that the UNPC tumor cell phenotype may contribute to T cell activation in this microenvironment.

Another member of the tumor necrosis factor receptor family, CD40, is expressed on B cells, interdigitating and follicular dendritic reticulum cells, some carcinomas, and non-neoplastic basal epithelial cells.[33,34] Its ligand (CD40-L) is expressed on activated T cells.[35,36] Signaling through CD40 can rescue germinal center B cells from apoptosis, partly

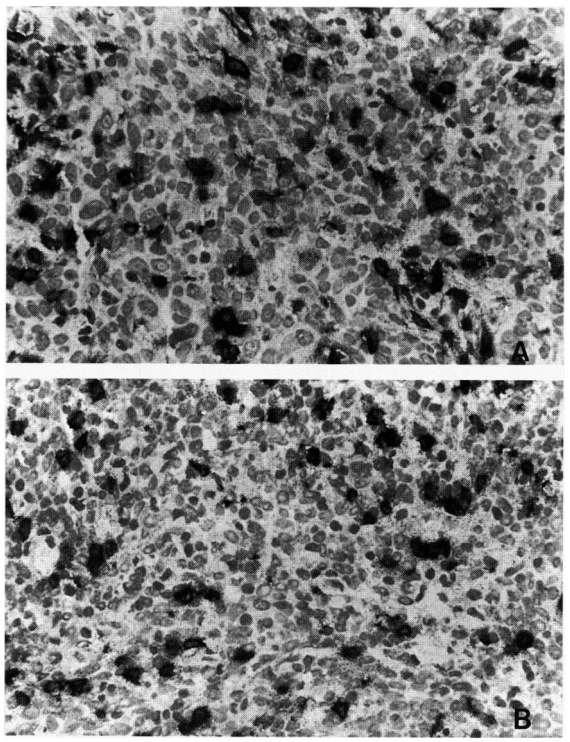

Figure 4. *Cells expressing CD4* (**A**) *and CD8* (**B**) *are seen in the lymphoid stroma of an undifferentiated NPC (case 1). APAAP method; hematoxylin counterstaining; magnification, × 350.*

through upregulation of Bcl-2.[33,37] Expression of CD40 in UNPCs has been reported previously[38] and is confirmed in this study. The presence of variable numbers of CD40-L positive T cells in UNPC biopsies demonstrated here suggests that UNPC tumor cells may be influenced through the CD40/CD40-L pathway. For this reason, we have also analyzed Bcl-2 expression in UNPCs. Almost all cases were Bcl-2 positive, confirming a previous report.[39] However, there was no absolute correlation between CD40 expression and the expression of Bcl-2 (Table 2), suggesting that stimulation through CD40 is not necessarily responsible for Bcl-2 expression in UNPCs. Although LMP-1 can upregulate Bcl-2 in B cells,[40] no such effect has been observed in epithelial cells *in vitro* (C. Dawson, L. S. Young, unpublished observation), which agrees with the lack of correlation between LMP-1 and Bcl-2 expression in UNPCs observed in this study. As both CD40 and Bcl-2 are expressed in normal basal epithelial cells in the nasopharynx, the detection of these proteins in UNPCs may simply reflect the undifferentiated phenotype of the tumor cells.

T cell activation requires recognition of an antigenic epitope presented through an appropriate major histocompatibility complex. In addition, T cell activation involves a co-stimulatory signal provided through CD28, a member of the immunoglobulin superfamily.[41,42] Signaling through CD28 in conjunction with T cell receptor engagement can upregulate the production of several cytokines, eg, interleukin-2.[43] Whereas CD28 is constitutively expressed in T cells, a related molecule, CTLA-4, has been identified in activated T cells.[44] Both CD28 and CTLA-4 are ligands for the B7 group of molecules (B7–1 (CD80), B7–2 (CD86), and B7–3) expressed on antigen-presenting cells.[41,45,46] It has been reported that the CD80 MAb used in our study, BB-1, binds to an epitope that is shared between B7–1 and B7–3 and that B7–3 may be expressed in keratinocytes.[47] CD28 and B7 appear to be of pivotal importance in T cell activation. Prevention of CD28 signaling by anti-B7 MAbs in the presence of cyclosporin A has been shown to induce clonal anergy.[48] On the other hand, transfection of B7 into B7 negative nonimmunogenic tumor cells may augment T-cell-mediated tumor rejection.[49] Thus, expression of B7 molecules in some UNPCs is unexpected. To our knowledge, this is the first demonstration of these antigens in carcinomas. B7 expression has been detected in the Jijoye Burkitt's lymphoma cell line, which harbors a transforming strain of EBV, but not in its daughter cell line, P3HR-1, which carries a nontransforming strain of the virus and lacks LMP-1.[50] Thus, it appears that in B cells B7 expression may be linked to functional activities of EBV. Interestingly, the expression of B7 antigen(s) in UNPCs appears to correlate with the detection of LMP-1. Thus, 5 of the 6 LMP-1 positive cases also expressed at least one member of the B7 family, whereas none of the 14 LMP-1 negative cases showed B7 expression. It has been suggested that CD70 expression in UNPCs may be linked to LMP-1 expression.[6,17] However, CD70 is expressed in most UNPCs, many of which are LMP-1 negative.[6,17] Thus, members of the B7 family represent the first phenotypic marker whose expression in UNPCs is associated with the detection of LMP-1.

B7 expression has been demonstrated in most Hodgkin's disease cases in which it is thought to contribute to the accumulation of lymphocytes.[51,52] Although this may also play a part in some UNPCs, it is clearly not the only explanation for the presence of a lymphoid stroma as B7 expression is seen only in a proportion of cases. It has been shown that in B cells and in Hodgkin's disease-derived cell lines, B7 expression may be upregulated by signaling through CD40.[52,53] As most UNPCs are CD40 positive, but only a minority expresses B7 molecules, it seems unlikely that CD40 ligation is sufficient to trigger B7 expression in this cellular environment. It is tempting

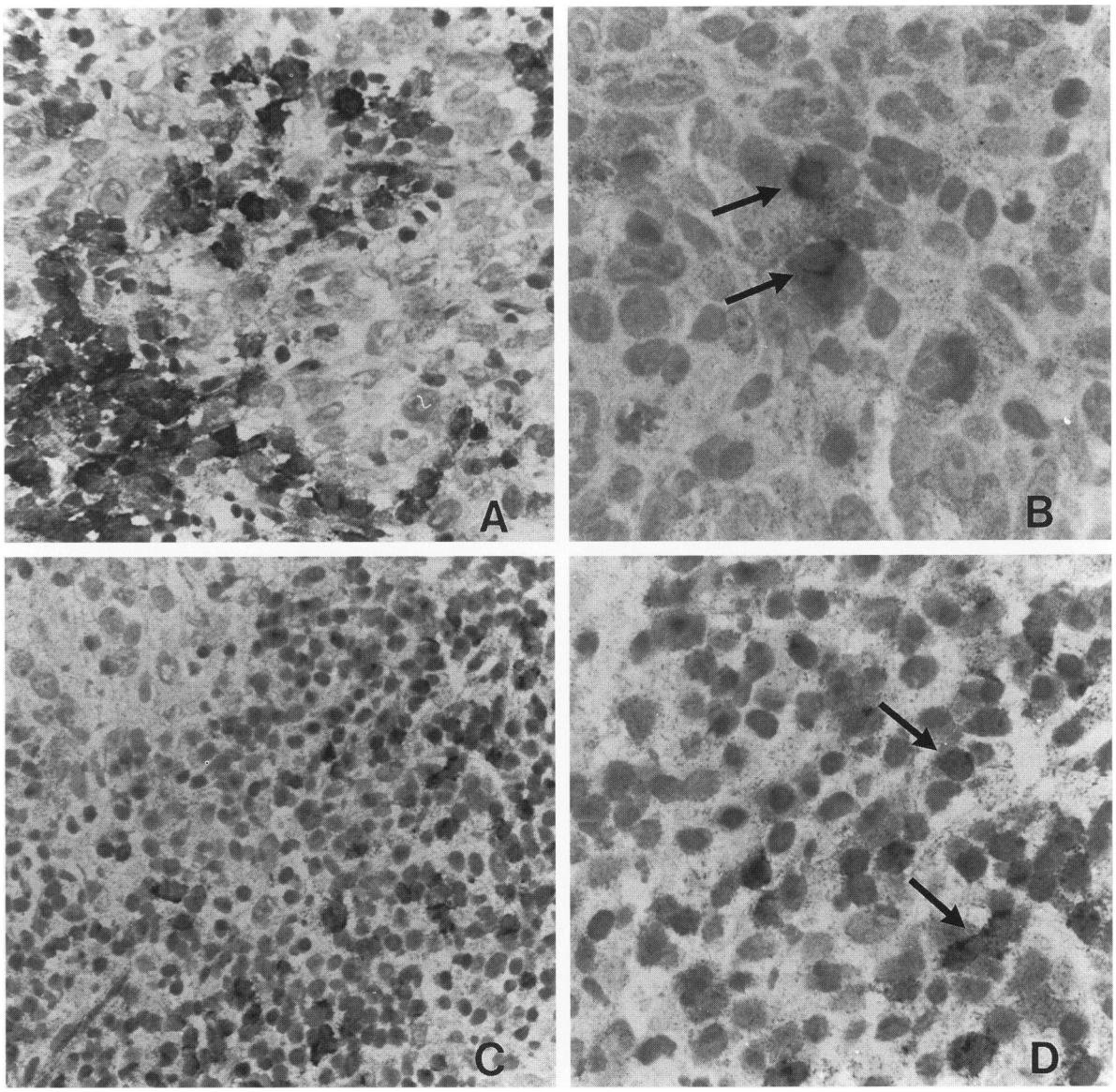

Figure 5. *Many CD27-expressing infiltrating cells are noticed in the lymphoid stroma of an undifferentiated NPC* (A; *case 4*). *Scattered lymphoid cells were noticed expressing CD28* (B; *case 11*, arrows) *and the CD40-L* (C *and* D; *case 12*, arrows). *APAAP method; hematoxylin counterstaining; magnification,* × *350* (A *and* C) *and* × *700* (B *and* D).

to speculate that in UNPCs LMP-1 expression may complement CD40 function to induce B7 expression.

In summary, our findings raise the possibility that in UNPCs tumor cells and infiltrating lymphocytes exchange signals involved in T cell activation and tumor cell growth. This supports the idea that interaction with T lymphocytes is a requirement for tumor cell growth at least in certain stages of UNPC development.[24] Additional studies of UNPC biopsies particularly with respect to the expression of cytokines in tumor cells and tumor-infiltrating lymphocytes will be required to clarify further the interrelationship between these two cellular compartments.

Acknowledgments

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